Tumor-Antigen–Binding Bispecific Antibodies for Cancer Treatment

Ulrich H. Weidle,[a] Roland E. Kontermann,[b] and Ulrich Brinkmann[a]

Bi- and multispecific antibody derivatives (bsAbs) can be considered as the next generation of targeted biologics for cancer therapy. The general concept of bsAbs is a physical connection of recombinant antibody-derived entities with at least two binding specificities. This generates bsAbs that bind at least two antigens or epitopes, thus altering their binding functionalities and specificities in comparison to "normal" antibodies. Most bsAbs are produced as recombinant proteins, either as large IgG-like proteins that contain Fc regions, or as smaller entities with multiple antigen-binding regions but without Fc. Application of bsAbs in experimental cancer therapy currently includes molecules that bind different cell surface proteins to achieve more complete blockage of proliferative or angiogenesis-associated pathways. This approach of blocking more than one pathway component, or to simultaneously hit complementing pathways, also may limit potential escape mechanisms of cancer cells. BsAbs also are applied in the clinic as vehicles to deliver immune effector cells and/or cytokines to tumors.

The concept of bispecific antibody derivatives that target multiple antigens was demonstrated more than 20 years ago by Sherie Morrison and colleagues.[1] Further development of Bi- and multispecific antibody derivatives (bsAbs) initially made rather slow progress mainly due to technical hurdles. Today, however, progress in protein design and production technologies has led to the availability of many different recombinant protein formats suitable for generation of bsAbs.[2–16] Recombinant bsAb formats can be assigned to two general classes: Fc-containing bsAbs (selected examples are shown in Figure 1A) and bsAb derivatives without Fc regions (selected examples are shown in Figure 1B). BsAbs that contain Fc are rather large molecules (> 100,000 kd) and frequently have a long serum half-life. The long serum half-life of these molecules is mediated by size (which minimizes renal clearance) and predominantly by Fc-mediated FcRn binding and antibody recycling. The "IgG-derived molecules" are usually produced in mammalian cells applying the same or similar production technologies as those that are established for the production of recombinant monospecific antibodies. Compatibility of bsAb generation with established upstream and downstream processes for production of "normal" IgGs can facilitate drug development.

A variety of Fc-containing bsAb formats are currently available that have binding modules for additional antigen recognition attached to N- or C-termini of IgG scaffolds. These include additional single-chain or disulfide stabilized Fvs or Fabs fused to IgGs, resulting in tetravalent molecules with bivalent binding specificity.[3–5] In a similar manner, combinations of single-chain variable fragments (scFvs) and scFabs placed upon IgGs enable the production of multivalent (hexa- or even octavalent) molecules that can recognize three (or more) different antigens.[6]

Dual-variable domain immunoglobulins are composed in a similar manner, having extra VH and VL domains of a second specificity placed upon the

[a]Roche Pharmaceuticals Research and Early Development (pRED), Discovery Oncology (UHW) and Large Molecule Research (UB), Roche Innovation Center Penzberg, Germany.
[b]Institute of Cell Biology and Immunology, University of Stuttgart, Stuttgart, Germany.
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Address correspondence to Ulrich Brinkmann, PhD, Roche Pharmaceuticals Research and Early Development (pRED), Roche Innovation Center Penzberg, Im Nonnenwald 2, D-82372 Penzberg, Germany. E-mail: ulrich.brinkmann@roche.com
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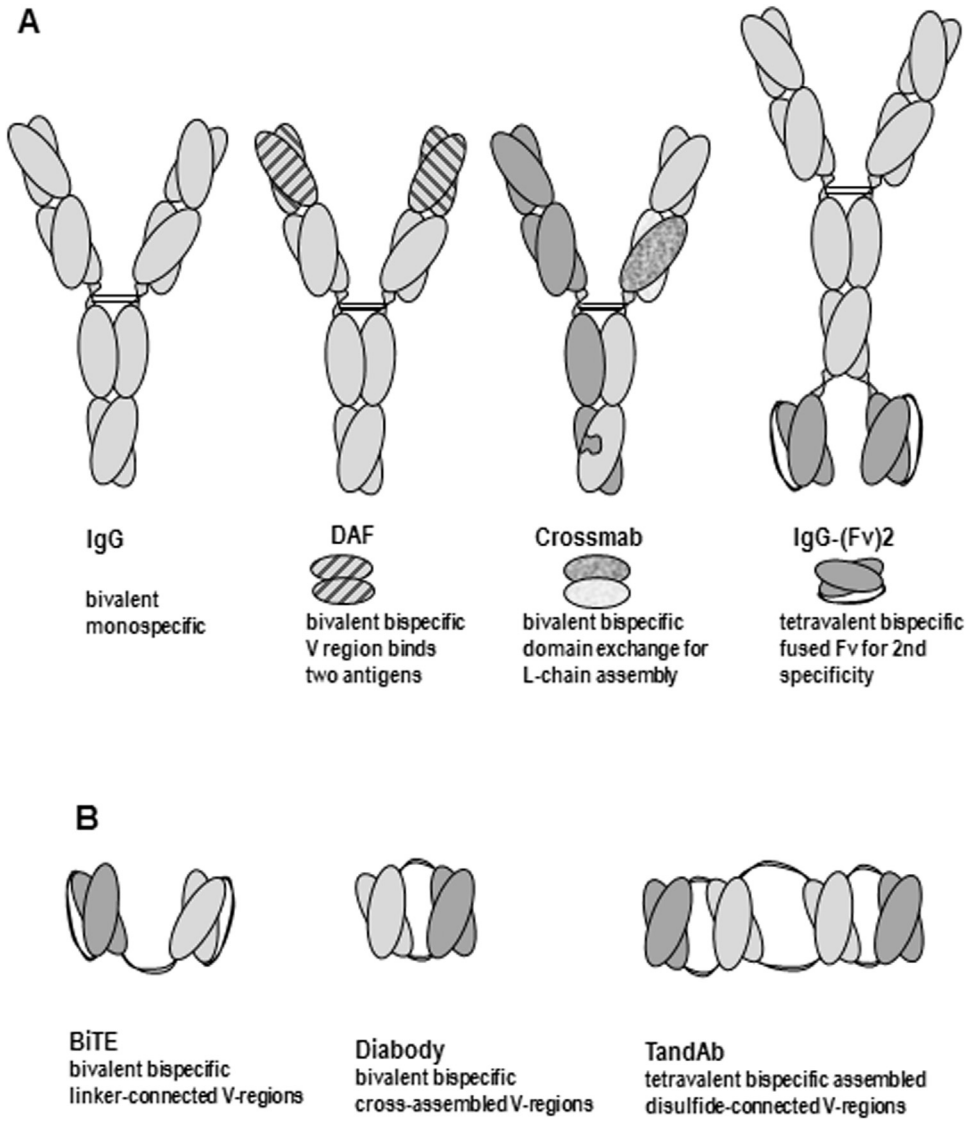

Figure 1. Design and composition of bsAbs. Bispecific antibodies can be large IgG-derived molecules containing Fc regions (A), or smaller derivatives without Fc regions (B).

N-termini of the V domains of IgGs.[7,8] Bispecific "mini-antibodies" are smaller than whole IgG-derived bsAbs and are composed of single-chain variable fragments (scFv) that are fused to the C-termini of the CH3 regions (CH3-scFv) and/or to the hinge region (scFv-hinge-Fc) of an antibody. Another IgG-derived format is the trivalent bsAb, which contains disulfide stabilized variable domains fused to the C-terminus of CH3 domains of IgGs. These bsAb derivatives whose additional binding sites are stabilized by interchain disulfides can bind one antigen in a bivalent and another antigen in a monovalent manner.[9,10]

Due to the bivalency of IgGs, bsAbs also can be designed without fusing extra binding entities to the IgG scaffold. Such molecules possess two binding arms that recognize different antigens.

Display technologies can be applied to evolve variable domains to recognize two unrelated antigens.[11] Recombinant antibodies with such variable domains are "normal" IgGs, but still with bispecific functionality because they can bind two antigens with each V-domain being occupied once. Other IgG-derived bsAbs that enable monovalent (and simultaneous) binding of different antigens are generated by forced heavy (H)-chain heterodimerization, combined with technologies that assure attachment of the correct light (L)-chain to its respective H-chain. Antibody H-chain heterodimer formation frequently can be achieved via application of the "knobs-into holes" technology that was pioneered quite early by Genentech.[12] BsAbs with knob-into-holes chains contain replacements of small amino acids with larger ones in one of their CH3 domains ("knob") and replacements of smaller amino acids ("hole") in the corresponding domain of the other H-chain. A similar principle is applied for modulating H-chain

heterodimerization by strand-exchange engineering (SEED)[13] using sequence exchanges between IgG1 and IgA to create non-identical hetero-dimerizing chains. L-chain mispairing must also be avoided to generate homogenous preparations of bispecific IgGs. The use of a "common light chain" principle that combines two binders that share one light chain but still have separate specificities[14] is one option for that. The recently developed CrossMab technology,[15,16] which avoids non-specific L-chain mis-pairing by exchanging CH1 and CL domains in the Fab of alf of the bispecific antibody is another robust technology to generate homogeneous bsAbs. BsAbs that are composed of such cross-over domains fully retain the binding specificity and affinity (and correct VL–VH chain pairing) of their parent antibodies.

BsAbs WITHOUT Fc REGIONS

BsAbs that do not carry Fc regions are frequently of a smaller size than Fc-containing antibody derivatives. They are frequently produced in yeast or bacterial expression systems, and hence cannot be purified by technologies that are "standard" for the production of IgGs. Most of bsAbs without Fc have a short serum half-life due to their small size (renal clearance) and due to the lack of interaction with the neo-natal Fc receptor.[17] On the other hand, tissue penetration of such small proteins molecules may be better compared to large IgG-like entities. The pharmacokinetics of small bsAbs can be modulated and serum half-life prolonged by conjugation with polymers such as polyethylene glycol,[18] or by fusion to albumin binding entities.[17,19]

Bispecificity is achieved by connecting individual binding modules such as scFvs or Fabs of different specificities to each other via flexible linker peptides. The connection of the different binding partners is either achieved by recombinant protein fusion, or by attachment of heterodimerization (and/or trimerization) functionalities.

One clinically applied example for bsAbs without Fc regions are BiTEs, bispecific T-cell engagers,[20] which are generated by fusing two scFvs to each other by a flexible linker peptide. Diabodies are another well-established and robust technology to generate bispecific binders lacking constant region sequences.[21,22] They contain two VH and two VL domains from two different antibodies, connected with short linker peptides in a manner that permits only the association of the correct VH-VL heterodimers. In diabodies, the VH of the first binder is linked to the VL of the second binder, which is co-expressed with the VH of the second antibody linked to VL of the first antibody. A modification of the diabody technology generates tetravalent tandem antibodies (TandAbs).[21] These molecules assemble by dimerization of a protein that contains four antibody variable H- and L-chains in an orientation that again prevents "wrong" intramolecular pairing. Another similar modification results in dual affinity retargeting molecules (DARTs), antibody derivatives that are stabilized by disulfide bonds but otherwise apply a similar design concept as diabodies.[23] Many other concepts that are applied to generate small bsAb derivatives were recently summarized.[2] One example is the "dock-and-lock" principle, which is based on heterodimerization of cyclic adenosine monophosphate (cAMP)-dependent protein kinase A and A-kinase anchoring protein[24] and is used to design dimerization and docking domains. These domains are linked to Fab fragments to assemble first a divalent Fab fragment, and in turn creating another docking site for binding to an attachment sequence incorporated into a second protein, resulting in the formation of a trivalent complex.

BsAbs IN CANCER THERAPY—RECRUITMENT OF IMMUNE EFFECTOR CELLS

Technical hurdles had hampered the development of bsAbs in the past. Although many promising molecules of different formats are currently in explorative or preclinical stages, still rather few are in clinical development. BsAbs that are currently in the clinic or in clinical development are listed in Table 1. These include molecules that bind different tumor-associated antigens (dual targeting) to achieve more complete blockage of proliferative or angiogenesis-associated pathways, or molecules for targeted delivery of immune effector cells and/or cytokines to tumors.

"First-generation" multispecific antibody preparations that have been applied in the clinic are trifunctional bsAbs developed by Trion Pharma (Munich, Germany). The technology for producing these (non-human) bsAbs has been quite unusual because Rat/mouse quadromas were used as expression vehicles. These quadromas generate IgG-like bsAbs with a correct H/L-chain pairing because species-specific chains preferentially assemble with each other.[25] The bsAbs are a hybrid of mouse IgG2a and rat IgG2b. They have three functionalities as they target a tumor antigen, they recruit T-effector cells via CD3 binding, and they can activate natural killer cells, monocytes/macrophages, and dendritic cells via binding of Fc to Fcγ-receptors.[26] A series of bsAbs were generated with this technology, including bsAbs that target epithelial cell adhesion molecule (EpCAM), HER2, CD20, or gangliosides GD2 and GD3.[27–33] All of these showed activity in preclinical

Table. 1. Bispecific Antibodies in Clinical Development

Target Combination	bsAb	Format	Status	Reference
EpCAMx CD3 (xFcγR)	Catumaxomab	Large with Fc quadroma-bsAb	Approved 2007 malignant ascites, phase II ovarian, gastrointestinal, non-small cell lung cancer, breast cancer; phase I/II peritoneal carcinomatosis	64
CD20 x CD3 (xFcγR)	Bi 20, FBTA-05	Large with Fc quadroma-bsAb	Phase I non-Hodgkin lymphoma	64
CD30xCD16,	AFM-13	Without Fc TandAb	Phase I Hodgkin lymphoma	65
CD3xCD19	Blinatumomab AMG-103, MT-103	Small without Fc BiTE	Phase II non-Hodgkin lymphoma (DLBCL), acute lymphocytic leukemia	66
CD3xEpCAM	Solitomab, MT-110, AMG-110	Small without Fc BiTE	Phase I solid tumors	67
CD3xCEA	MT-111, MEDI-565	Small without Fc BiTE	Phase I advanced gastrointestinal	68
CD3xPSMA,	BAY-2010112, MT-112.	Small without Fc BiTE	Phase I, prostate cancer	69
HER2xHER3,	MM-111,	Without Fc scFv-(mHSA)-scFv	Phase I breast, stomach, and esophageal cancer	55
HER3xIGF-1R	MM-141	Large with Fc IgG+scFv	Phase I colid tumors	70
EGFRxHER3,	RG-7597 MEHD-7945A	Large with Fc two-in-one IgG	Phase II metastatic colorectal cancer, head and neck stem cell cancer	60
Ang2xVEGF-A	RG-7221	Large with Fc CrossMab	Phase I advanced solid tumors, avastin-resistant tumors	63

evaluations. Catumaxomab, a very potent anti-EpCAM/anti-CD3 bsAb, was brought into the clinic[27] and efficiently eliminated tumor cells in patients with ovarian carcinoma and prevented accumulation of ascites. The molecule has an acceptable safety profile, with very small doses (5–20 μg) being administered four to five times by intraperitoneal infusions within 9–13 days. Due to the non-humanness of the bsAb, moderate anti-mouse or anti-rat responses were observed in most patients; however, this did not affect the efficacy of the treatment. Another bsAb produced by the same technology is ertumaxomab (anti-HER2/anti-CD3), which has progressed into clinical studies in patients with metastatic mammary carcinoma.[33]

Another technical approach to bsAbs for recruitment of immune effector cells is the development of bipecific T-cell Engagers (BiTEs).[34,35] In contrast to the quadroma-derived IgG-derivatives which contain Fc regions, BiTEs are small and have a short serum half-life. They consist of two scFvs connected by a flexible linker[35–37] with one Fv directed at a tumor cell surface antigen, and the second scFv binding the CD3ε subunit of the T-cell receptor complex. BiTEs are produced as recombinant proteins in *Escherichia coli*. Potent anti-tumor cell cytotoxicity has been observed for many BiTEs,[38] inducing specific target cell lysis in cell culture assays at concentrations ranging between 10 pg/mL and 10 ng/mL.[39] BiTEs elicit repeated rounds of target cell lysis by T cells at very low effector/ target (E/T) cell ratios,[40] with T-cells killing being mediated by membrane perforation and subsequent induction of granzymes and apoptosis.[41] It is important to note that the affinity of the antibody directed against the tumor-associated antigen is much higher than that directed against CD3.[35] A series of BiTEs that bind different tumor cell surface antigens have been generated, including molecules that target CD19, EpCAM, HER2, carcinoembryonic antigen (CEA), ephrin A2 (EphA2), CD33, and melanoma-associated chondroitin sulfate proteoglycan (MCSP). Blinatumomab[36] is an anti-CD19/anti-CD3 BiTE with excellent potency leading to tumor eradication in many preclinical models.[42,43]. In patients, blinatumomab successfully depletes chronic lymphocytic leukemia (CLL) B cells in the presence of autologous T cells. This included patients that had been pretreated with different

chemotherapy regimens.[44] Tumor regression also was observed in non-Hodgkin lymphoma patients.[45] Blinatumomab is applied at low doses (as low as 0.005–0.06 mg/m^2), the relatively short half-life of BiTEs also requires an appropriate dosing schedule and formulation to ensure continuous activation of T cells against target cells. Other BiTEs that are in clinical studies are composed of EpCAM, prostate-specific membrane antigen (PSMA), or CEA-binding molecules combined with CD3-binding modules (Table 1).

TandAbs are tetravalent, bispecific fusion proteins that were developed for the recruitment of immune effector cells.[46] In contrast to BiTEs, TandAbs have four binding sites, and they are larger than BiTEs but do not carry Fc domains. Nevertheless, they have a longer serum half-life than BiTEs because they are larger than the cut-off size for glomerular filtration. AFM13 is a CD30- and CD16A-binding Tandab that has shown good preclinical activity by recruitment of natural killer cells and macrophages.[47] AFM13 (CD30xCD16A) entered clinical studies for the treatment of Hodgkin lymphoma.

BsAbs DIRECTED AT RECEPTOR-TYROSINE KINASES

The Her family of receptor-tyrosine kinases (RTKs) has four members, HER1/EGFR, HER2, HER3, and HER4. Various heterodimer constellations of these receptors (HER1, 2, 3) are receptors for epidermal growth factor (EGF)-related ligands. Ligand binding, in turn, can induce RTK activity, trigger intracellular signaling cascades, and promote tumor growth.[48] For example, HER2 is overexpressed in up to 30% of breast carcinomas, which correlates with a shorter time to relapse and shorter overall survival.[49] HER3 also is involved in breast cancer development and progression, as phosphorylation of HER3 was observed in *HER2*-amplified breast cancer.[50] HER2–HER3 heterodimerization leads to breast cancer cell proliferation and is involved in transformation.[51] Monospecific antibodies that bind to and interfere with signaling of Her family members, such as cetuximab (erbitux, HER1) or trastuzumab (herceptin, HER2) are successfully applied in cancer therapy. Due to extensive cross-talk between HER family members and signaling events, BsAbs directed at multiple members of the Her family are developed with the rationale that addressing more than one member of this family might improve therapeutic efficacy.[52,53] For example, HER2-binding trastuzumab and pertuzumab by themselves are rather poor inhibitors of ligand-induced HER3 activation,[54,55] but bsAb-mediated targeting of both HER2 and HER3 can stop the growth of cancer cell lines with amplified *HER2*.[56] One bsAb in phase I study that binds HER2 as well as HER3 is MM-111. It is composed of two antagonistic scFvs that bind HER2 and HER3, which are linked to each other as well as to a modified human serum albumin to modulate the pharmacokinetics of the Fc-less molecule.[55] MM-111 binds to and forms complexes with HER2 and HER3, and potently inhibits the proliferation of tumor cells in vitro and in vivo, with efficacy depending on HER2 expression. Another bsAb in phase I study is MM141, which bind HER3 as well as insulin-like growth factor (IGF)-1R, another RTK that is not a member of the HER family. MM141 is composed of scFvs that are added to the Fc regions of an IgG. The rationale for combining IGF-1R and HER3 binding specificities into one bsAb is the observation that IGF-1R signaling also is deregulated in many types of cancer.[57–60] Thus, simultaneous inhibition of HER3- and IGF-1R signaling could be a valid therapeutic strategy to overcome escape of tumor cells.

A bsAb that binds to EGFR (HER1), as well as to HER3 that is in a phase II trial is RG7597 (MEDH7945A). This molecule is an IgG whose binding regions have been evolved to permit effective binding to EGFR, as well as to HER3 with good affinities (1.9 and 0.4 nmol/L, respectively).[61] As a human IgG1, MEHD7945A interferes not only with EGFR and HER3 signaling, but—due to the presence of Fc—also can exert immune effector functions. The rationale for combining HER1- and HER3 binding specificities into one bsAb is the observation that complete inhibition of MAPK and AKT signaling (and subsequent growth arrest of pathway addicted cells) can be achieved when EGFR and HER3 are blocked simultaneously. Thus, simultaneous inhibition of HER1 and HER3 could be a valid therapeutic strategy to improve tumor therapy.[60]

BsAbs IN CANCER THERAPY—INTERFERENCE WITH TUMOR ANGIOGENESIS

Antibody-mediated interference with tumor vascularization can be applied for the treatment of solid tumors. Tumor angiogenesis is controlled and modulated by multiple angiogenic factors. In consequence, tumors may become resistant towards anti-angiogenic treatments that inactivate only one factor (eg, vascular endothelial growth factor [VEGF]) by activation of alternative pathways.[61,62,63] Simultaneous depletion of multiple factors or interference with multiple angiogenesis pathways may therefore improve anti-angiogenic therapy by reducing potential escape mechanisms, eg, due to activation of alternative pathways.[62,63] One anti-angiogenic bsAb currently in phase I clinical trials binds to and

inhibits the functionality of VEGF-A, as well as angiopoietin-2 (Ang2). VEGF is the target of monospecific antibody bevacizumab (avastin) and binds to VEGF receptor 1 (VEGFR1) and VEGFR2, which in turn promotes and modulates angiogenesis. Ang2 is a ligand of the Tie-2 kinase, which also contributes to and modulates tumor angiogenesis. The bsAb that binds VEGF-A and Ang-2 is an IgG-like "CrossMab" with a shape and size and pharmacokinetic parameters of normal IgGs.[15,16,61] Preclinical evaluation revealed better activity of the bsAb in comparison to the monospecific VEGF-A–binding (bevacizumab) or Ang-2–binding antibody. The bispecific CrossMab administered at the equivalent dose in terms of binding sites mediated the best tumor growth inhibition compared to monospecific parent antibodies or combinations thereof.[61]

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